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ANALYSIS OF TIAZOFURIN-INDUCED DNA DAMAGE IN HUMAN WHOLE BLOOD CELLS USING AN IN VITRO COMET ASSAY

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ANALIZA DNK OŠTEĆENJA IZAZVANOG TIAZOFURINOM U HUMANIM ĆELIJAMA PUNE KRVI PRIMENOM IN VITRO KOMET TESTA

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ABSTRACT

Objective. Inosine 5'-monophosphate dehydrogenase (IMPDH) activity in cancer cells is increased. Tiazofurin selectively inhibits the activity of IMPDH, and it has been granted for the treatment of different cancers and new viral diseases. Its widespread use was limited because exposure to tiazofurin under certain circumstances was found to have a higher frequency of severe non-hematologic toxicity. Therefore, the objective of this study was to examine genotoxic action and inducement of DNA damage of tiazofurin using the comet assay.

Methods. The ability of tiazofurin to induce DNA damage was evaluated using single-cell gel electrophoresis (SCGE) technique/comet assay. Human whole blood cells were exposed to three final concentrations of tiazofurin (1 μM/mL, 2 μM/mL, and 5 μM/mL) for 30 min in vitro.

Results. Our results indicate that tiazofurin produced a significant level of DNA damage on whole blood cells after 30 min of exposure vs. control. All tested concentrations were significantly comet-forming, in a concentration-dependent manner.

Conclusion. Our investigation on the tiazofurin-treated cells and their relationship to the formation of DNA damage demonstrated that the genotoxic effect was induced after exposure to tiazofurin under described conditions.

Key words: *DNA damage; mutagenicity tests; comet assay*

SAŽETAK

Cilj. Aktivnost inozin 5'-monofosfat dehidrogenaze (IMPDH) povećana je u ćelijama karcinoma. Tiazofurin selektivno inhibira aktivnost IMPDH i odobren je za lečenje različitih karcinoma i novih virusnih bolesti. Njegova široko rasprostranjena upotreba bila je ograničena jer je utvrđeno da je izloženost tiazofurinu pod određenim okolnostima imala veću incidencu ozbiljne nehematološke toksičnosti. Stoga je cilj ove studije bio da se pomoću komet testa ispita genotoksično delovanje i izazivanje DNK oštećenja tiazofurinom.

Metode. Sposobnost tiazofurina da izazove DNK oštećenje procenjena je primenom elektroforeze DNK pojedinačnih ćelija (SCGE) / komet testa. Ćelije pune krvi su bile izložene trima konačnim koncentracijama tiazofurina (1 μM/mL, 2 μM/mL, and 5 μM/mL) tokom 30 minuta in vitro.

Rezultati. Naši rezultati ukazuju na to da je tiazofurin proizveo značajan nivo DNK oštećenja na ćelijama pune krvi nakon 30 minuta izlaganja u odnosu na kontrolu. Sve ispitivane koncentracije su dovele do značajnog nastanka kometa, pri čemu je nivo oštećenja rastao s koncentracijom.

Zaključak. Naše istraživanje ćelija tretiranih tiazofurinom i njihova reakcija na izazivanje DNK oštećenja pokazalo je da je tiazofurin ispoljio genotoksični efekat pod opisanim uslovima.

Ključne reči: *DNK oštećenje; testovi mutagenosti; komet test*

INTRODUCTION

Inosine 5'-monophosphate dehydrogenase (IMPDH) catalyzes the committed step in *de novo* guanine nucleotide biosynthesis (1,2). Fast-growing cells are in high demand for guanine nucleotides, which is why IMPDH activity is increased in viral infections, tumors, and rapidly proliferating tissues (3,4). In various cell lines, IMPDH inhibitors induce apoptosis and differentiation (5,6) and are used in antiviral and immunosuppressive chemotherapy (4).

Tiazofurin (2-β-D-ribofurasylthiazole-4-carboxamide, TR) selectively inhibits the activity of IMPDH (7,8). The inhibition of IMPDH induced by TR is due to its active metabolite, thiazol-4-carboxamide adenine dinucleotide (TAD), an analog of nicotine-amid adenine dinucleotide (NAD^+). TAD binds tightly to the NADH site, thus inhibiting the activity of IMPDH (9,10). Though similar to that of NADH, the inhibitory effect of TAD is three orders of magnitude higher than the one of natural NADH (10). Since IMPDH is increased in malignant and leukemia cells (7,8), potent IMPDH inhibitory properties

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and antitumor activity (11,12) lead to the examination of TR and its derivates for use in the treatment of cancer (13-15). TR-induced apoptosis in K562 and HL-60 myeloid cells, as well as in Jurkat and MOLT-4 T lymphoid cell lines (16,17). TR induced complete hematological remissions in patients with end-stage acute non-lymphocytic leukemia or myeloblastic crises of chronic myeloid leukemia (18). TR was granted orphan drug status for the treatment of chronic myelogenous leukemia, though widespread use was limited due to its toxicity (4, 9,19). Non-hematologic toxicity of TR included serum transaminase elevations, headache, and signs of central nervous system, cardiac and ocular toxicity (20). Studies revealed that TR induced an increase in the frequency of micronuclei (MNi) in peripheral blood lymphocytes, correlated to the time of exposition (21). An investigation of the genotoxic effects of TR stereoisomers showed them as far less genotoxic than TR (22). Therefore, the current study aimed to examine genotoxic action and assess the level of DNA damage in human whole blood cells (WBC) caused by tiazofurin through *in vitro* single cell gel electrophoresis technique (comet assay).

MATERIAL AND METHODS

2.1. Subjects

Peripheral blood samples were collected in heparinized containers from three healthy donors aged between 21 and 55. Donors did not receive any therapy or medications, take any dietary supplements, consume alcohol, or smoke during the study.

2.2. Study Design

Tiazofurin (CAS no. 60084-10-8, Biosynth, Staad, Switzerland) was used in this study, diluted in phosphate-buffered saline (PBS, Torlak Institute of Immunology and Virology, Belgrade, Serbia) to final concentrations of 1 μM/mL, 2 μM/mL, and 5 μM/mL. Three final concentrations of tiazofurin were determined according to the literature data (21, 23-25). To evaluate the genotoxic potential of tiazofurin in human peripheral blood samples, they were incubated for 30 minutes at 37°C with chosen concentrations of tiazofurin. Hydrogen peroxide (H_2O_2, CAS no. 7722-84-1, ZORKA Pharma, Sabac, Serbia), a well-known oxidant, was used as a positive control. 50 μM H_2O_2 was added to the cells for 20 min at 4°C. Negative controls were treated with PBS at 37°C for 30 min. The experiments for all three samples were performed in duplicate.

2.3. The Single Cell Gel Electrophoresis Assay

The lysis, denaturation, electrophoresis, and staining in the alkaline comet assay were performed as described by Singh et al. (26). The whole blood samples (6 μL per slide) were suspended in 0.67% low-melting-point (LMP) agarose (Sigma-Aldrich, St. Louis, MO) and pipetted onto previously coated microscope slides. After solidifying at 4°C for 5 min and the removal of the coverslips, the cell suspensions were treated as described above. Following the treatments, the slides were covered with a layer of 0.5% LMP agarose and left to solidify at 4°C for 5 min. Afterwards, the slides were placed in a pre-chilled lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X100, and 10% dimethylsulfoxide, adjusted with NaOH to pH 10) and left to stay overnight at 4°C. After the unwinding and electrophoresis (in electrophoresis buffer at pH 13 - 10 M NaOH, 200 mM EDTA) the next day, the slides were rinsed - two times for 10 min in neutralization buffer (0.4 M Tris, pH 7.5) and one time in distilled water. Staining was done with ethidium bromide (20 μg/L, Sigma-Aldrich, St. Louis, MO). The comets were analyzed at a magnification of 100x, on an Olympus BX 50 microscope (Olympus Optical Co., GmbH, Hamburg, Germany), equipped with a mercury lamp HBO (50 W, 516–560 nm, Carl Zeiss Inc.), connected to a computer via CCD camera, allowing appropriate zoom (Figure 1). The comets were visually scored and, depending on the extent of DNA migration, classified into five categories according to Anderson et al. (27): A - no damage; B - low-level damage; C - medium level damage; D - high-level damage; and E - total damage. 50 randomly selected comets were scored on each of 2 slides per subject (100 in total per subject). DNA damage was characterized as DNA migration over 5% (B+C+D+E comet categories) for all three subjects.

2.4. Statistical Analysis

The results were obtained as the mean and standard error (SEM), for n=3. Data were processed in the GraphPad Prism 6.0 software, using analysis of variance (one-way ANOVA) and Tukey's posthoc test for determination of statistical significance. A difference at $p<0.05$ was considered statistically significant.

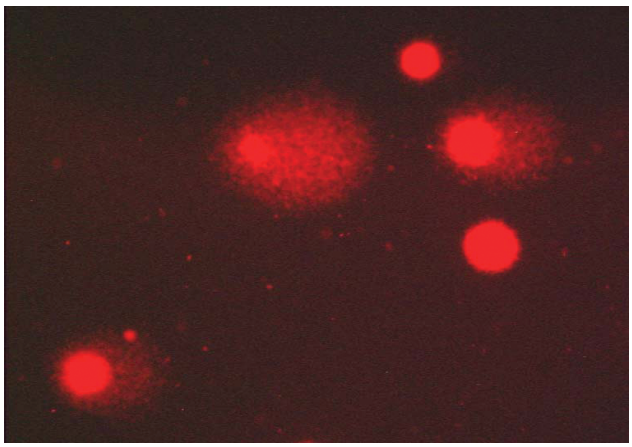

Figure 1. The example of tiazofurin-exposed cells, showing DNA migration after treatment

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RESULTS

Figure 2 summarizes the effect of TR in WBC (mean of three experiments from different donors). Comet assay analysis revealed that the tested range of concentrations (1 μM/mL, 2 μM/mL, and 5 μM/mL) induced a significant level of DNA damage when compared to untreated cells (incubation with PBS). TR induced a concentration-related increase in DNA migration. Moreover, the effect of 5 μM/mL TR showed a statistically significantly greater level of DNA damage than 1 μM/mL TR. Table 1 represents the effect of TR expressed by the percentage of comet categories among affected WBC. Low and medium levels of DNA migration (B and C comet categories) are mostly present in cells treated with tested concentrations of TR, as well as in the ones treated with controls. Figure 3 shows the effect of TR on WBC of individual donors, considering a wide age range between them. The same trend is visible as in Table 1.

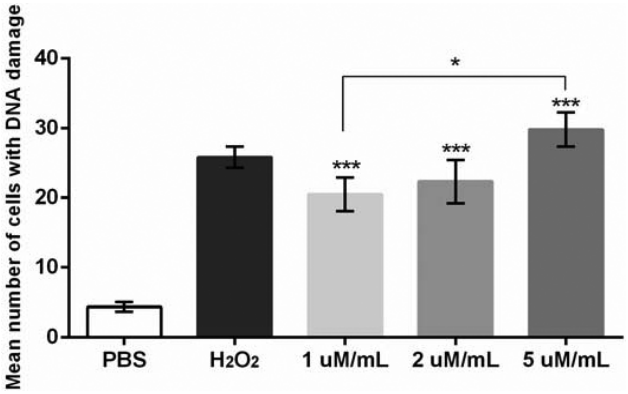

*Figure 2. Genotoxic effect of TR in tested concentrations (1 μM/mL, 2 μM/mL, and 5 μM/mL) after 30 min of treatment at 37°C, positive control with H2O2, and negative control with PBS. Bars represent mean number of cells with DNA damage ± SEM, for n = 3. ***p < 0.0001 vs. PBS; *p < 0.05, 1 μM/mL vs. 5 μM/mL*

Table 1. Relationship between the effect of TR and the degree of DNA damage. The values represent the percentage of comet categories among affected WBC: B - low-level damage, C - medium level damage, D - high-level damage, and E - total damage

Variable	B	C	D	E
PBS	46.2	50.0	0.0	3.8
H_2O_2	31.6	49.7	16.1	2.6
1 μM/mL	22.8	64.2	9.8	3.3
2 μM/mL	18.7	74.6	6.0	0.7
5 μM/mL	14.0	64.8	10.6	10.6

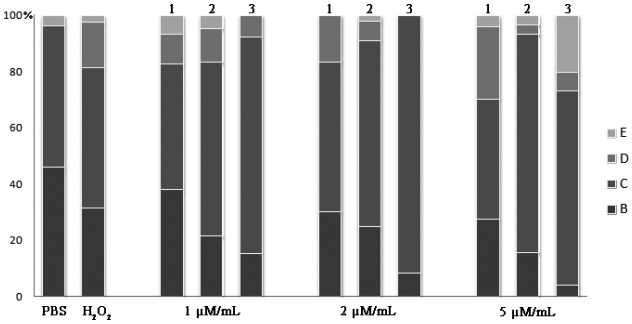

Figure 3. Relationship between the effect of TR and the degree of DNA damage, represented as values for individual donors (1-22 years, 2-21 years, 3-55 years). Values are expressed as the percentage of comet categories among affected WBC: B - low-level damage, C - medium level damage, D - high-level damage, and E - total damage

DISCUSSION

In vitro and *in vivo* studies found that TR has an antiproliferative effect on tumor cells (12,28). TR was also tested in Lewis lung carcinoma and MM-96 human melanoma cells, where resistance to tiazofurin was quickly developed (25). Despite the fact that a good correlation was detected between biochemical parameters and clinical response in leukemic patients, especially the ones with chronic myeloid leukemia, phase I trials were discontinued because of toxicity (3). An analysis of the patient database showed that exposure to TR under certain circumstances may result in a higher frequency of severe non-hematologic toxicity (20).

Our results indicate that TR induces DNA damage in WBC after an *in vitro* exposure period of 30 min. A significant increase in DNA damage is detected by the comet assay after treatment with 1 μM/mL, 2 μM/mL, and 5 μM/mL TR in comparison to incubation with PBS. Another test suitable to monitor genotoxicity in cells, the micronucleus test (MN), demonstrated that the proliferation potential of cells was significantly reduced, especially after a prolonged treatment with TR (21). Nevertheless, the same study revealed that the clastogenic properties of TR were evidenced in the treatment period of 17 h (21). A study by Berger et al. (23) showed that continuous exposure of cells to tiazofurin for 24 h and 48 h resulted in a dose-dependent increase in DNA strand breaks, measured by alkaline elution. TR-induced strand breaks appeared more resistant to repair than those induced by 75-150 rad of γ-irradiation. Results obtained in our study reveal that even short-term exposure to TR can cause damage of hereditary material, unlike the ones obtained by the MN test. On the other hand, not only that our data are consistent with the study which showed dose-dependent increases in DNA damage induced by TR, but they show that the highest tested concentration (5 μM/mL) induced a statistically significant increase compared to the lowest concentration (1 μM/mL).

Besides its ability to act as a DNA strand-disrupting substance, some studies indicated that TR may be useful in

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chemotherapy combinations, to potentiate the effects of agents with similar effects. Dexamethasone, a medicine that induces DNA strand breaks and depletion of NAD pools against steroid-sensitive mouse lymphoma cells was potentiated by TR (29). The same team previously found that TR interferes with the synthesis of NAD, reduces the cellular content of NAD, and serves as a weak inhibitor of poly (ADP-ribose) polymerase, an enzyme that requires NAD for normal DNA repair processes (23).

In summary, a genotoxic effect related to exposure to TR in WBC was revealed using the comet assay, one of the most accepted assays used to detect DNA damage. Our investigation of the TR-treated cells and relationship to the formation of DNA damage demonstrates the dose-dependent DNA damage induced by TR, with lower-grade damage being more present in affected cells.

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